PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

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NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

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in its capacity as elected Office

Date of mailing (day/month/year) 05 December 2000 (05.12.00)	
International application No. PCT/CA00/00483	**Applicant's or agent's file reference** 10662-86PCT
International filing date (day/month/year) 27 April 2000 (27.04.00)	**Priority date** (day/month/year) 28 April 1999 (28.04.99)
Applicant SMITH, Lawrence, C. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

15 November 2000 (15.11.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

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PATENT COOPERATION TREATY

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
10662-86PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/CA 00/ 00483	27/04/2000	28/04/1999
Applicant		
UNIVERSITE DE MONTREAL et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

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   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

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2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

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   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

   [ ] as suggested by the applicant.

   [X] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 00/00483

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/00 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BORDIGNON,V. & SMITH L.C.: "TELOPHASE ENUCLEATION: AN IMPROVED METHOD TO PREPARE RECIPIENT CYTOPLASTS FOR USE IN BOVINE NUCLEAR TRANSFER " MOLECULAR REPRODUCTION AND DEVELOPMENT, vol. 49, no. 1, January 1998 (1998-01), pages 29-36, XP000910821 US,NEW YORK the whole document ---	1-38
X	NOUR MS, TAKAHASHI Y : "Preparation of young preactivated oocytes with high enucleation efficiency for bovine nuclear transfer. " THERIOGENOLOGY , vol. 51, no. 3, February 1999 (1999-02), pages 661-666, XP000934249 the whole document ---	1-38

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
3 October 2000	11/10/2000

Name and mailing address of the ISA	Authorized officer
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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 07669 A (CAMPBELL KEITH HENRY STOCKMAN ;ROSLIN INST EDINBURGH (GB); WILMUT) 6 March 1997 (1997-03-06) the whole document	1-38
A	WILMUT I ET AL: "VIABLE OFFSPRING DERIVED FROM FETAL AND ADULT MAMMALIAN CELLS" NATURE,GB,MACMILLAN JOURNALS LTD. LONDON, vol. 385, no. 6619, 27 February 1997 (1997-02-27), pages 810-813, XP002067035 ISSN: 0028-0836 cited in the application the whole document	1
A	SUN F. Z. ET AL.: "NUCLEAR TRANSPLANTATION IN MAMMALIAN EGGS AND EMBRYOS" CURRENT TOPICS IN DEVELOPMENTAL BIOLOGY,ACADEMIC PRESS, NEW YORK, NY,,US, vol. 30, 1995, pages 147-166-166A-167-176, XP000921085 ISSN: 0070-2153 the whole document	1
P,X	BAGUISI A, ET AL.: "Production of goats by somatic cell nuclear transfer." NAT BIOTECHNOL, vol. 17, no. 5, May 1999 (1999-05), pages 456-461, XP000891364 the whole document	1

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 00/00483

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9707669 A	06-03-1997	AU 716956 B	09-03-2000
		AU 6831096 A	19-03-1997
		BR 9610034 A	21-12-1999
		CA 2229568 A	06-03-1997
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		CZ 9800608 A	15-07-1998
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		NZ 316149 A	28-10-1999
		PL 325331 A	20-07-1998

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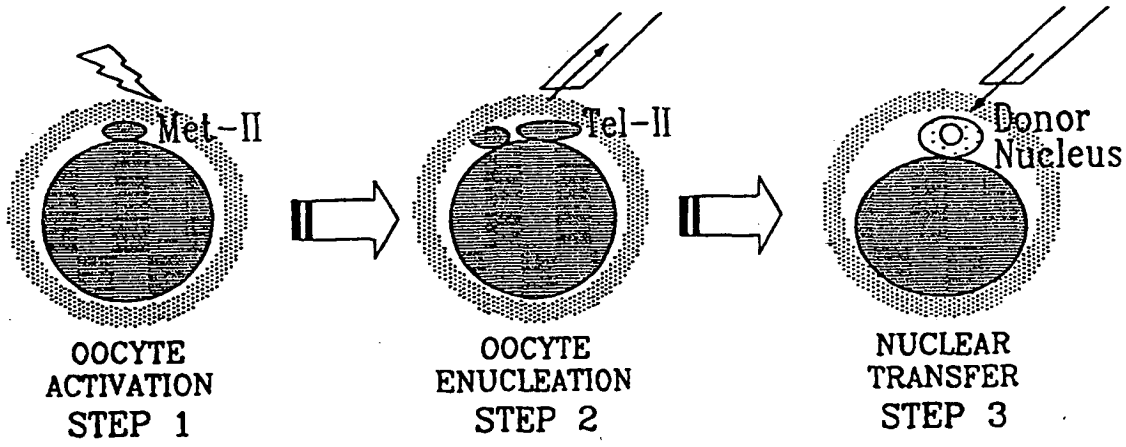
(54) Title: TELOPHASE ENUCLEATED OOCYTES FOR NUCLEAR TRANSFER



(57) Abstract

The present invention relates to an improved method for obtaining an enucleated host oocyte for transferring nuclei from embryonic, germinal and somatic cells with the objective of cloning or multiplying mammals, and to a method of reconstituting a non–human embryo.

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TELOPHASE ENUCLEATED OOCYTES FOR NUCLEAR TRANSFER

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The present invention relates to an improved method for obtaining an enucleated host oocyte for transferring nuclei from embryonic, germinal and somatic cells with the objective of cloning or multiplying mammals, and to a method of reconstituting an animal embryo.

(b) Description of Prior Art

The technique of nuclear transfer has been widely used to multiply embryos by transferring blastomere nuclei from early-stage embryos into enucleated oocytes. This technique enables an increase in the yield of embryos produced from parents of top genetic value, enabling to accelerate the annual genetic gain within an animal population. Nuclear transfer has also been used with nuclei from cell lines derived from embryonic (Campbell et al., 1996, *Nature* **380**:64-66), fetal and adult tissue (Wilmut et al., 1997, *Nature* **385**:810-813). By using nuclei from an unlimited source, nuclear transfer from cell lines enables not only the production of a larger number of genetically identical offspring but also an opportunity for modifying the genetic characteristic of cells *in vitro* prior to the production of live offspring, enabling the production of transgenic mammals. Moreover, the use of cells from adult animals for nuclear transfer, either directly or through previous *in vitro* passage, enable the multiplication (cloning) of animals of known phenotypes.

Basically, the nuclear transfer technique requires a donor nucleus to provide the genetic material of choice and a host oocyte to provide the cytoplasm that plays a role in reprogramming the

nucleus to support embryo development. With the nuclear and cytoplasm sources in hand, three main steps are required to reconstruct an oocyte by nuclear transfer. First, host oocytes need to be enucleated to remove all nuclear genetic material. This step is usually performed by microsurgical removal of the chromosomes from either a metaphase plate or pronuclei. Second, donor nuclei need to be introduced into the oocyte (nuclear transfer). This step is normally obtained by fusing the membranes of the nuclear donor cell and the host oocyte. However, nuclear transfer can also be obtained by traversing the oocytes plasma membrane and microinjecting the nucleus directly into the host cytoplasm. Finally, non-activated host oocytes need awakening from their meiotic arrest (oocyte activation). This step can be achieved by exposing the oocyte to a physical stimulus, such as temperature changes or an electric shock, or exposing the oocyte to chemical agents, such as ethanol or exogenous calcium. The order in performing each of the steps above can vary in different situations and may have an important effect on the ability of the reconstructed oocyte to undergo further development.

In mice, oocyte enucleation was performed after fertilization by visualizing and removing the pronuclei by microsurgery. This enucleation technique is less efficient in other mammals due to the higher density of cytoplasm resulting in poor visualization of pronuclei. Moreover, attempts to use pronuclear-stage enucleated oocytes led invariably to poor developmental rates when using cleavage stage blastomeres as nuclear donors. Improved development after nuclear transfer was achieved initially in sheep (Willadsen,S. 1986, *Nature* **320**:63-65) and later in other mammals using host oocytes that had not been activated at the time of

fusion. However, a problem remained that metaphase stage chromatin cannot be visualized easily by microscopy in most mammals. Willadsen (Willadsen, S.1986, *Nature* **320**:63-65) proposed an enucleation procedure in which sheep oocytes were blindly divided into halves either containing or not the first polarbody. To avoid a large loss of cytoplasm during enucleation, this procedure was later improved by using a DNA vital stain (Bisbenzimide; Hoechst) and ultraviolet (UV) irradiation to check whether the MII plate after removal of small portions of cytoplasm. The most common procedure of oocyte enucleation is to expose secondary oocytes to bisbenzimide, blindly remove a cytoplasmic fragment surrounding the first polarbody and then expose the oocyte to UV to ascertain whether enucleation was correctly performed. On average this procedure correctly enucleates between 60 to 80 percent of oocytes. Another possible limitation of this procedure is that oocytes are exposed both to UV irradiation and Hoechst 33342 that have been shown to have detrimental effects on the cytoplasm (Smith,L. 1993 *J. Reprod. Fert.* **99**:39-44).

As mentioned above, host oocytes are able to support better development after nuclear transfer when compared to pronuclear-enucleated host zygotesIt has already been shown that MII-stage enucleated oocytes either aged or activated before fusion support better development. The problem of using young non-activated oocytes is caused by incompatibilities between the cell cycle stages of the nuclear donor cell and the host cytoplasm. Metaphase arrested secondary (MII) oocytes have high levels of a Maturation Promoting Factor (MPF), a cellular activity that is responsible for maintaining the chromatin condensed without a nuclear envelop. When blastomere interphase-stage nuclei

containing decondensed chromatin are introduced into an MII oocyte, MPF leads to a rapid breakdown of the nuclear membrane and premature chromosome condensation (PCC). However, PCC is believed to be detrimental only when induced during the DNA synthesis stage (S-phase) of cell cycle. This is particularly problematic when using donor nuclei from blastomeres since these undergo S-phase for most time in between cell divisions. On the other hand, enucleated oocytes that have been activated or aged before fusion to nuclear donor cells have lower levels of MPF and, therefore, do not cause PCC.

With the exception of blastomeres, most other cell types have longer gaps both before (G1-phase) and after (G2-phase) the S-phase and, therefore, are less susceptible to the harmful effects of S-phase PCC when fused to a MII oocytes. Because high MPF levels cause the breakdown of the nuclear membrane, MII stage host oocytes are believed to facilitate interactions between donor nuclei and putative oocyte cytoplasmic 'factors' required for reprogramming the chromatin of nuclei derived from cells further advanced in differentiation. Several examples in the literature report on the advantages of passaging further differentiated donor nuclei in non-activated MII oocytes before activating the reconstructed oocyte. In cattle, nuclei from an embryonic cell line supported significantly higher yield of blastocyst development and more 30d pregnancies when fused to enucleated oocytes 4 h before activation. In mice, significantly more embryos reconstructed with cumulus cell nuclei developed to the blastocyst stage by exposing the donor nucleus to MII cytoplasm for between 1 and 6 h before activation (Wells et al. 1999, *Biol. Reprod.* **60**:996-1005). Moreover, no fetal development or live offspring was obtained when using with simultaneous activation and

fusion. Furthermore, other reports using differentiated cell lines have used host oocytes that were either activated after or concurrently with introducing the donor nucleus (Cibelli et al. 1998, *Nature Biotechnol.* **16**:642-646; Wilmut et al. 1997, *Nature* **385**:810-813). Therefore, the prevalent theory in the field of cloning by nuclear transfer is that a period of reprogramming in the cytoplasm of an inactivated oocyte is required to obtain success when using donor nuclei from cells other than embryonic blastomeres.

It would be highly desirable to be provided with an improved method for obtaining an enucleated host oocyte for transferring nuclei from embryonic, germinal and somatic cells with the objective of cloning or multiplying mammals.

It would be highly desirable to be provided with an improved method of reconstituting an animal embryo.

SUMMARY OF THE INVENTION

The present invention described below is contrary to current knowledge in that we are teaching use of an activated oocyte as recipient for nuclei derived from cells from embryonic and somatic cell lines.

One aim of the present invention is to provide an improved method for obtaining an enucleated host oocyte for transferring nuclei from embryonic, germinal and somatic cells with the objective of cloning or multiplying mammals.

Another aim of the present invention is to provide an improved method of reconstituting a non-human embryo.

In accordance with the present invention there is provided a method of preparing an enucleated host

oocyte for transferring nuclei from embryonic, germinal or somatic cells, which comprises the steps of:

a) activating oocyte by artificial means; and

b) enucleating the activated oocyte when the activated oocyte is undergoing the expulsion of a second polarbody or when the activated oocyte has recently expelled second polarbody (Tel-II); and

c) transferring nuclei from embryonic, germinal or somatic cells into the enucleated oocyte of step b), wherein embryonic cells are cultured prior to nuclei transfer.

The germinal or somatic cells are cultured prior to nuclei transfer.

The oocyte of step a) has a first polarbody and the artificial means is chemical means, such as ethanol or ionomycin.

Step b) may be performed after oocytes are cultured for a period of time sufficient to allow for extrusion of a second polarbody.

Step b) may be performed with oocytes in a medium with cytosqueletal inhibitors.

Step b) may be effected by microsurgically removing the second polar with about one tenth of the cytoplasm surrounding the second polarbody.

The preferred oocyte is a secondary (M-II) oocyte.

In accordance with the present invention, there is provided a method of reconstituting a non-human embryo, which comprises the steps of:

a) activating oocyte by artificial means;

b) enucleating the activated oocyte when the activated oocyte is undergoing the expulsion of a second polarbody or when the activated oocyte

has recently expelled second polarbody (Tel-II);

c) transferring a diploid nucleus in the enucleated oocyte to obtain a reconstructed oocyte with a diploid chromosomal content; and

d) culturing *in vitro* the reconstructed oocyte and/or transferring the reconstructed oocyte into a reproductive tract of a suitable surrogate mother to enable development into a non-human embryo.

In accordance with the present invention, there is provided a method for production of a transgenic non-human embryo, which comprises the steps of:

a) transfecting cultured cells selected from the group consisting of embryonic, germinal and somatic cells with a desired DNA construct;

b) activating oocyte by artificial means;

c) enucleating the activated oocyte when the activated oocyte is undergoing the expulsion of a second polarbody or when the activated oocyte has recently expelled second polarbody (Tel-II);

d) transferring a diploid nucleus extracted from the transfected cells of step a) in the enucleated oocyte to obtain a reconstructed oocyte with a diploid chromosomal content; and

e) culturing *in vitro* the reconstructed oocyte and/or transferring the reconstructed oocyte into a reproductive tract of a suitable surrogate mother to enable development into a non-human embryo.

The non-human embryo may develop into a non-human animal.

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 illustrates a schematic protocol of the technique of telophase enucleation for nuclear transfer.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method of producing embryos by nuclear transplantation from embryonic, germinal and somatic cells lines. Nuclear transfer procedures have invariably initiated with the enucleation of host oocyte. The enucleation procedure is followed by one of the following: (a) activation followed by fusion; (b) concurrent activation and fusion; or (c) fusion followed by activation. Whereas the procedure in which oocytes are (a) enucleated, activated and then fused is used mostly for embryonic blastomeres, most techniques applied for further differentiated donor nuclei use the procedure where oocytes are enucleated, (b) fused and activated concurrently or (c) fused and later activated. Although the different approaches in the nuclear transfer procedure have been described previously (U.S. Patent No. 4,994,384; U.S. Patent No. 5,057,420; U.S. Patent No. 5,843,754 and International Patent applications Nos. PCT/GB96/02098, PCT/US98/00002, PCT/US98/12800, PCT/US98/12806, and PCT/US97/12919), the present invention describes a sequence of steps in the nuclear transfer procedure that is novel (Fig. 1).

As illustrated in Fig. 1, Step 1 involves the activation of secondary (M-II) oocytes by artificial means. Step 2 is performed shortly after activation when the oocyte is undergoing the expulsion or recently expelled the second polarbody (Tel-II). Step 3 relates to the transfer of a nucleus from any source with the

purpose of reconstructing the oocyte with a diploid chromosomal content.

Step 1 (oocyte activation)

Oocytes are obtained either *in vivo* or *in vitro* and cultured in maturation medium. After maturation, oocytes are denuded of cumulus cells and those with a first polarbody are parthenogenetically activated by chemical means using ethanol or ionomycin. After activation, oocytes are cultured for a few hours to allow for extrusion of the second polarbody.

Step 2 (oocyte enucleation)

After activation, oocytes can be placed in medium with cytosqueletal inhibitors to facilitate microsurgery. Only oocytes with a second polarbody extruded or partially extruded are used. Approximately one tenth of the cytoplasm surrounding the second polar body is microsurgically removed with the second polarbody.

Step 3 (nuclear transfer)

After enucleation, a single cell containing a diploid nucleus is introduced into the enucleated oocyte either by cell fusion or microinjection (nuclear transfer). The reconstructed oocyte is then cultured *in vitro* and/or transferred into the reproductive tract of a suitable surrogate mother to enable further development.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE 1

Telophase Enucleation

Follicles with 2 to 8 mm diameter were aspirated from bovine slaughterhouse ovaries. Oocytes with a homogeneous cytoplasm and several layers of cumulus cells were selected and placed in maturation within 1 h from follicular aspiration. At 28 h after maturation oocytes were denuded of cumulus cells and those with a first polarbody were used in the experiment. Oocytes were exposed to 7% ethanol for 5 min, washed and placed in maturation medium for different periods. At 1 h before microsurgery, oocytes were placed in cytochalasin B and positioned for micromanipulation. Oocytes undergoing extrusion or already with extruded second polarbodies had 10% of their cytoplasmic volume removed together with the second polarbody. After microsurgery, oocytes were fixed in 10% formalin, stained with 5 µg Hoechst 33342 and observed under UV epi-fluorescence. Oocytes without any chromatin were considered successfully enucleated. Most oocytes were successfully enucleated when micromanipulated at the times examined (Table 1). Because the efficiency of this enucleation technique is high, checking of oocytes with DNA stain and UV light is not necessary. Significantly lower percentages of enucleation was obtained when blindly removing using the position of the first polarbody to aspirate 30% of the surrounding cytoplasm in oocytes at metaphase (59%) at 24 h from the beginning of *in vitro* maturation.

Table 1

Successful telophase enucleations as performed at different times after exposure to a stimulus to parthenogenetically activate secondary oocytes

	Time after activation			
	3 h	4 h	5 h	Total
Number manipulated	37	38	43	118
Successful enucleation (%)	36 (97%)	37 (97%)	40 (93%)	113 (96%)

Example 2

Nuclear transfer with morula-stage blatomeres

Bovine secondary oocytes were matured *in vitro* and enucleated using the technique described above (telophase enucleation). Morula-stage embryos were disaggregated and individual blastomeres were inserted into the perivitelline space of enucleated oocytes. Fusion between the membranes of blastomeres and oocytes was obtained with an electric pulse that causes fusion between the membranes of the donor and recipient cells. The electrical parameters used were double 60 µsec pulses of 1.5 KVolts per cm. After fusion the embryos were cultured for 7 days in the presence of Menezo's B2 medium supplemented with 10% fetal calf serum.

Table 2

Fusion and development of bovine oocytes reconstructed with nuclei from morula-stage blastomeres recovered 5 days after IVF

	Number	Fused	Blastocyst	No. nuclei
Telophase II (%)	215	129 (58%)	49 (38%)	126±11
Metaphase II (%)	248	151 (60%)	24 (16%)	84±9

Example 3

Nuclear transfer with non-starved bovine ES cells

Bovine embryo stem (ES)-like cells were obtained from day 8 blastocyst stage embryos produced entirely *in vitro*. ICMs were platted onto mitomycin-inactivated mouse fibroblasts. Established ES-like lines were disaggregated by short exposure to trypsin using a narrow pipette. Isolated cells were placed in the perivitelline space of enucleated oocytes and exposed to an electric pulse that causes fusion between the membranes of the donor and recipient cells. The electrical parameters used were double 100 µsec pulses of 1.5 KVolts per cm. Electrical stimulation was performed as soon as possible after placing the nuclear donor cell in the perivitelline space to obtain better fusion results. After fusion the embryos are cultured for 7 days in the presence of Menezo's B2 medium supplemented with 10% fetal calf serum.

Table 3

Fusion and development of bovine oocytes reconstructed with nuclei from ES-like cells exposed to 5% of FCS

	Number	Fused	Cleaved	Blastocyst
Telophase II (%)	38	11 (30%)	5 (45%)	3 (27%)
Metaphase II (%)	33	12 (36%)	2 (17%)	1 (8%)

Example 4

Nuclear transfer with serum-starved bovine ES cells

Bovine embryo stem (ES)-like cells were cultured in medium with 0.5% FCS for 5 days before micromanipulation. As described above, ES-like cells were disaggregated, placed in the perivitelline space of enucleated oocytes and exposed to an electric pulse to cause fusion between the membranes of the donor and

recipient cells. After fusion the embryos are cultured for 7 days in the presence of Menezo's B2 medium supplemented with 10% fetal calf serum.

Table 4

Fusion and development of bovine reconstructed with nuclei from bovine ES-like cells exposed (starved) to low concentrations (0.5%) of FCS

	Number	Fused	Cleaved	Blastocyst
Telophase II(%)	38	13(34%)	3(23%)	2(27%)
Metaphase II(%)	42	13(31%)	4(31%)	1(15%)

Example 5

Nuclear transfer with starved and non-starved bovine fetal fibroblasts

Bovine fetal fibroblast cells were recovered form day 50 fetuses and passaged in medium D-MEM with 10% FCS. Non-starved fibroblast cells were recovered during growth at 2 days after passaging. Serum starved cells were exposed to medium with 0.5% serum for 5 days before NT. NT was performed as described above.

Table 5

Fusion and development of bovine reconstructed with nuclei from bovine fetal fibroblast cells exposed for 5 days to low concentrations (0.5%) of FCS (starved) or to 5% FCS for 20 h after seeding (non-starved)

	Serum starved			Non-starved		
	Number	Fused	Blast.	Number	Fused	Blast.
Telophase II (%)	69	52 (75%)	2 (4%)	105	67 (64%)	9 (13%)
Metaphase II (%)	60	39 (65%)	9 (24%)	114	92 (81%)	12 (13%)

Example 6

Nuclear transfer with starved and non-starved bovine fetal fibroblasts transfected with a GFP construct

Bovine fetal fibroblast cells were recovered form day 50 fetuses and passaged in medium D-MEM with 10% FCS. The fetal fibroblast cells were transfected with a constructs containing the CMV/eGFP gene (plasmid pGREEN LANTERN-1, Life Technologies). This construct contains the reporter gene Green Fluorescence Protein (GFP) from Aequorea victoria jellyfish, which codes for a naturally fluorescent protein requiring no substrate for visualization. The GFP used is "humanized" (ie., codon sequence) and mutated to contain threonine at position 65 to enhance fluorescence peaking. The advantage of using this fluorescent gene as a reporter being that it yields bright green fluorescence when living or fixed cells are illuminated with blue light and increases our sensitivity of detection. The plasmid contains the CMV immediate early enhancer/promoter upstream of the GFP gene, followed by SV40 t-intron and polyadenylation signal. NT was performed as described above.

Table 6

Fusion and development of bovine reconstructed with nuclei from bovine fetal fibroblast cells transfected with a GFP construct and starved for 4 days and transferred to metaphase stage-enucleated oocytes or cultured for 6 h after thawing and transferred to telophase stage-enucleated oocytes

	Number	Fused	Blastocyst
Telophase II(%)	187	131(71%)	15(11%)
Metaphase II(%)	209	169(81%)	23(14%)

Table 7

Post-implantation development of cloned blastocyts derived from GFP-positive fetal fibroblasts (Table 6)

	No Embryos	No Recipients	Non-returned	60 d positive	200 d positive	liveborn
Telophase II(%)	11	6	2	1	1	1
Metaphase II(%)	15		5	4	4	3

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method of preparing a reconstructed oocyte by transferring cell or nucleus from germinal or somatic cells into an enucleated host oocyte, which comprises the steps of:

a) activating said host oocyte by artificial or natural means; and

b) enucleating said activated host oocyte when said activated oocyte is undergoing the expulsion of a second polarbody or when said activated oocyte has recently expelled second polarbody (Tel-II);

c) transferring nucleus from germinal or somatic cells into said enucleated host oocyte of step b) to obtain a reconstructed oocyte.

2. The method according to claim 1, wherein said transferred cell or nucleus is at nuclear stage G0, G1, S, G2, or M.

3. The method of claim 1, wherein said germinal or somatic cells of step c) are cultured prior to nucleus transfer.

4. The method of claim 1, wherein said oocyte of step a) is a secondary oocyte (M-II) and said artificial means is physical or chemical means.

5. The method of claim 4, wherein said chemical means is ethanol or ionomycin.

6. The method of claim 4, wherein said physical means is selected from the group consisting of electrical means, thermal means, and irradiation technology.

7. The method of claim 1, wherein step b) is performed after oocytes are cultured for a period of time sufficient to allow for at least partial extrusion of a second polarbody.

8. The method of claim 1, wherein step b) is performed with oocytes in a medium with cytosqueletal inhibitors.

9. The method of claim 7, wherein step b) is effected by microsurgically removing said second polarbody with a portion of the cytoplasm containing chromosomes surrounding said at least partially extruded second polarbody.

10. A method of reconstituting a non-human embryo, which comprises the steps of:

a) activating oocyte by artificial or natural means;

b) enucleating said activated oocyte when said activated oocyte is undergoing the expulsion of a second polarbody or when said activated oocyte has recently expelled second polarbody (Tel-II);

c) transferring a diploid nucleus or a cell in said enucleated oocyte to obtain a reconstructed oocyte with a diploid chromosomal content; and

d) culturing *in vitro* said reconstructed oocyte and/or transferring said reconstructed oocyte into a reproductive tract of a suitable surrogate mother to enable development into a non-human embryo.

11. The method according to claim 10, wherein said transferred cell or nucleus is at nuclear stage G0, G1, S, G2, or M.

12. The method of claim 10, wherein said oocyte of step a) is a secondary oocyte (M-II) and said artificial means is physical or chemical means.

13. The method of claim 12, wherein said chemical means is ethanol or ionomycin.

14. The method of claim 12, wherein said physical means is selected from the group consisting of electrical means, thermal means, and irradiation technology.

15. The method of claim 13, wherein step b) is performed after oocytes are cultured for a period of time sufficient to allow for at least partial extrusion of a second polarbody.

16. The method of claim 15, wherein step b) is performed with oocytes in a medium with cytosqueletal inhibitors.

17. The method of claim 15, wherein step b) is effected by microsurgically removing said second polarbody with a portion of the cytoplasm containing chromosomes surrounding said at least partially extruded second polarbody.

18. The method of claim 17, wherein step c) is effected by introducing a single cell containing a diploid nucleus into said enucleated oocyte by cell fusion or by microinjection.

19. The method of claim 10, wherein said non-human embryo develops into a non-human animal.

20. A method for production of a transgenic non-human embryo, which comprises the steps of:

a) activating oocyte by artificial or natural means;

b) enucleating said activated oocyte when said activated oocyte is undergoing the expulsion of a second polarbody or when said activated oocyte has recently expelled second polarbody (Tel-II);

c) transferring a transgenic diploid nucleus extracted from a cell transfected with a desired DNA construct in said enucleated oocyte to obtain a reconstructed oocyte with a diploid chromosomal content; and

d) culturing *in vitro* said reconstructed oocyte and/or transferring said reconstructed oocyte into a reproductive tract of a suitable surrogate mother to enable development into a non-human embryo.

21. The method according to claim 20, wherein said transferred cell or nucleus is at nuclear stage G0, G1, S, G2, or M.

22. The method according to claim 20, which further comprises developing said non-human embryo into a fetus.

23. The method according to claim 22, which further comprises developing said fetus into an offspring.

24. The method of claim 20, wherein said non-human embryo develops into a non-human animal.

25. A transgenic embryo obtained according to the method of claim 20.

26. A transgenic fetus obtained according to the method of claim 21.

27. A transgenic offspring according to the method of claim 22.

28. A method of cloning a non-human animal by cell or nuclear transfer which comprises the steps of :

a) activating oocyte by artificial means;

b) enucleating said activated oocyte when said activated oocyte is undergoing the expulsion of a second polarbody or when said activated oocyte has recently expelled second polarbody (Tel-II);

c) transferring a diploid nucleus or a cell in said enucleated oocyte to obtain a reconstructed oocyte with a diploid chromosomal content; and

d) culturing *in vitro* said reconstructed oocyte and/or transferring said reconstructed oocyte into a reproductive tract of a suitable surrogate mother to enable development into a non-human embryo.

29. The method according to claim 28, wherein said transferred cell or nucleus is at nuclear stage G0, G1, S, G2, or M.

30. The method of claim 28, wherein said oocyte of step a) is a secondary oocyte (M-II) and said artificial means is physical or chemical means.

31. The method of claim 30, wherein said chemical means is ethanol or ionomycin.

32. The method of claim 30, wherein said physical means is selected from the group consisting of electrical means, thermal means, and irradiation technology.

33. The method of claim 28, wherein step b) is performed after oocytes are cultured for a period of time sufficient to allow for at least partial extrusion of a second polarbody.

34. The method of claim 30, wherein step b) is performed with oocytes in a medium with cytosqueletal inhibitors.

35. The method of claim 31, wherein step b) is effected by microsurgically removing said second polarbody with a portion of the cytoplasm containing chromosomes surrounding said at least partially extruded second polarbody.

36. The method of claim 32, wherein step c) is effected by introducing a single cell containing a diploid nucleus into said enucleated oocyte by cell fusion or by microinjection.

37. The method of claim 28, wherein said nucleus or cell of step c) is transgenic or non-transgenic.

38. The method of claim 28, wherein said non-human embryo develops into a non-human animal.

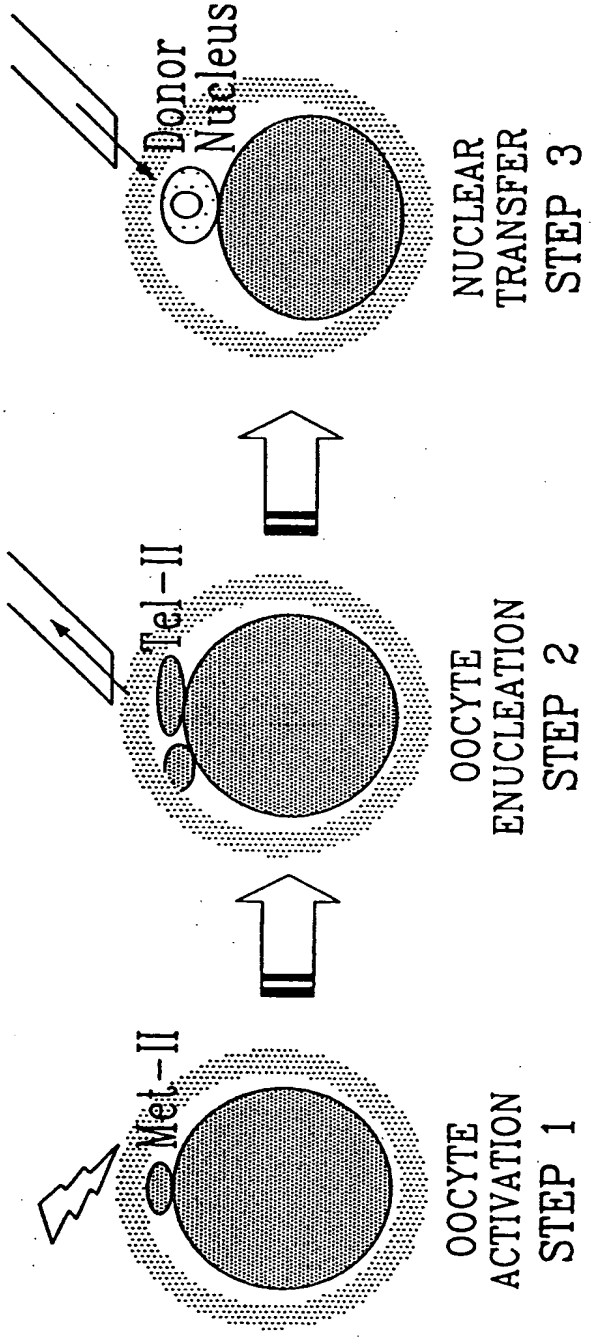

Fig-1

INTERNATIONAL SEARCH REPORT

Inter [redacted]onal Application No
PC[redacted] 00/00483

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/00 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BORDIGNON,V. & SMITH L.C.: "TELOPHASE ENUCLEATION: AN IMPROVED METHOD TO PREPARE RECIPIENT CYTOPLASTS FOR USE IN BOVINE NUCLEAR TRANSFER " MOLECULAR REPRODUCTION AND DEVELOPMENT, vol. 49, no. 1, January 1998 (1998-01), pages 29-36, XP000910821 US,NEW YORK the whole document ---	1-38
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3 October 2000	11/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Chambonnet, F

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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(71) Applicant *(for all designated States except US)*: UNIVERSITE DE MONTREAL [CA/CA]; 2900 Edouard–Montpetit, Montréal, Québec H3T 1J4 (CA).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: SMITH, Lawrence, C. [CA/CA]; 2950 Lafontaine, Saint–Hyacinthe, Québec J2S 2H9 (CA). BORDIGNON, Vilceu [CA/CA]; 2790 Sicotte, Saint–Hyacinthe, Québec J2S 2L5 (CA).

(74) Agent: SWABEY OGILVY RENAULT; Suite 1600, 1981 McGill College Avenue, Montréal, Québec H3A 2Y3 (CA).

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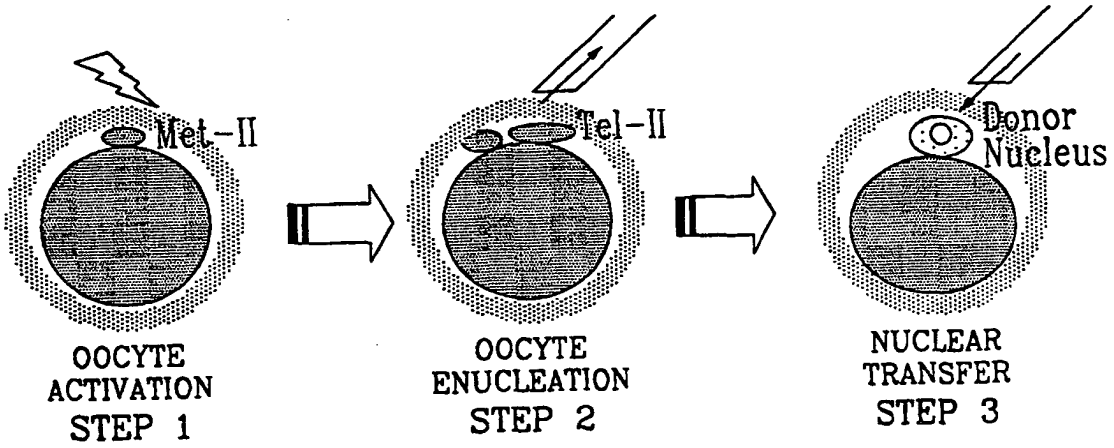
(54) Title: TELOPHASE ENUCLEATED OOCYTES FOR NUCLEAR TRANSFER



(57) Abstract

The present invention relates to an improved method for obtaining an enucleated host oocyte for transferring nuclei from embryonic, germinal and somatic cells with the objective of cloning or multiplying mammals, and to a method of reconstituting a non–human embryo.

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INTERNATIONAL SEARCH REPORT

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PCT/CA 00/00483

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/00 A01K67/027

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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3 October 2000	11/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Chambonnet, F

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/CA 00/00483

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 00/00483

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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PATENT COOPERATION TREATY

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(PCT Rule 47.1(c), first sentence)

To:
SWABEY OGILVY RENAULT
Suite 1600
1981 McGill College Avenue
Montréal, Québec H3A 2Y3
CANADA

Date of mailing (day/month/year) 02 November 2000 (02.11.00)	
Applicant's or agent's file reference 10662-86PCT EC	**IMPORTANT NOTICE**
International application No. PCT/CA00/00483	**International filing date** (day/month/year) 27 April 2000 (27.04.00)
Priority date (day/month/year) 28 April 1999 (28.04.99)	
Applicant UNIVERSITE DE MONTREAL et al	

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   02 November 2000 (02.11.00) under No. WO 00/65035

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra

Telephone No. (41-22) 338.83.38

Continuation of Form PCT/IB/308

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

Date of mailing (day/month/year)	IMPORTANT NOTICE
02 November 2000 (02.11.00)	
Applicant's or agent's file reference	**International application No.**
10662-86PCT	PCT/CA00/00483

The applicant is hereby notified that, at the time of establishment of this Notice, the time limit under Rule 46.1 for making amendments under Article 19 has not yet expired and the International Bureau had received neither such amendments nor a declaration that the applicant does not wish to make amendments.

IC

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70) 14

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
10662-86PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA00/00483	27/04/2000	28/04/1999
International Patent Classification (IPC) or national classification and IPC C12N15/00		
Applicant UNIVERSITE DE MONTREAL et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 8 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
15/11/2000	28.06.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Roscoe, R Telephone No. +49 89 2399 2554

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CA00/00483

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-15	as originally filed				

Claims, No.:

1-38	as received on	30/05/2001	with letter of	30/05/2001	

Drawings, sheets:

1/1	as originally filed				

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   ☐ the language of publication of the international application (under Rule 48.3(b)).
   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.
   ☐ filed together with the international application in computer readable form.
   ☐ furnished subsequently to this Authority in written form.
   ☐ furnished subsequently to this Authority in computer readable form.
   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:
   ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

☐ copy of the earlier application whose priority has been claimed.

☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	5, 32
	No:	Claims	1-4, 6-31, 33-37
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-38
Industrial applicability (IA)	Yes:	Claims	25-27
	No:	Claims	1-24, 28-38

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

I. Basis

The documents mentioned in the present written opinion / International Preliminary Examination Report are numbered as in the search report, i.e. D1 corresponds to the first document of the search report etc.

II. Priority

Priority could not be acknowledged for those claims which make reference to activation of oocytes by (i) natural means, (ii) physical means, (iii) by specific listed physical means, or to claims referring to specific cell-cycle stages (G0, G1, S...). The use of this terminology cannot be detected in the priority document, neither is it obviously derivable therefrom. The fact that it may be possible to infer this matter from the priority document is not sufficient to establish priority.

Only the following claims are thus entitled to priority from 28.04.99:

Claims 1, 3, 5, 7-9, 13, 15-18, 28, 31, 33-35, 37 and 38

V. Reasoned statement on Novelty, Inventive Step and Industrial Applicability

- Novelty (Art.33(2) PCT)

D1 discloses enucleation technique where oocytes are activated using ethanol. This is followed by microsurgical removal of the telophase-stage chromatin in a small volume of cytoplasm adjacent to the second polar body. Following enucleation, a single blastomere derived from an in vitro produced morulla was injected into the perivitelline space of the enucleated oocyte. Fusion of the membranes was performed by electrical pulsing. The reconstructed oocytes obtained by the new technique produced developmentally competent reconstructed oocytes. Technique suggested to be useful for research and practice of mammalian cloning. D1 relates specifically to cloning of animals using early embryonic blastomeres. Such cells can be considered as having the status of both germinal or somatic. Unspecified periods of culture can obviously not establish a difference between this prior art and the present application since

unless specific times are defined which clearly differentiate from the prior art, the unspecified periods have to be considered as an unclear and thus irrelevant technical feature.

D1 anticipates claims 10-13, 15-18, 20, 21, 25, 28-31, 33-37.

D2 discloses enucleation technique which differs from the D1 technique essentially in that sequential calcium ionophore and cycloheximide treatment are used to activate oocytes. Further, it is specified explicitly that both recipient ooplast and donor blastomeres are probably effectively in S-phase. Suggests that use method to produce large numbers of identical progeny. D2 does not only relate to metaphase II enucleation. D2 compares enucleation efficiency before and after oocyte activation. Already in the abstract it is stated that 100% of chromatin material was found adjacent to the second polar body after the activation. This is clearly referring to oocytes that have proceeded beyond metaphase II to the telophase II at which the extrusion of the second polar body is evident. Applicants attention is also drawn to the first two paragraphs of the results section.

D2 anticipates claims 10-12, 15-18, 20, 21, 25, 28-30, 33-37.

D3 discloses a different enucleation technique. However anticipates claims 25-27, since embryos / animals / offspring are not distinguishable whether produced by method involving enucleation at 1st or 2nd polar body.

D4 discloses production of transgenic sheep. Anticipates claims 25-27.

D6 is only relevant to claims other than 1, 3, 5, 7-9, 13, 15-18, 28, 31, 33-35, 37 and 38 (see section on priority). D6 discloses electrofusion of transgenic somatic goat cells with oocytes which have been enucleated at Tel-II stage after activation by (i) calcium, (ii) ethanol. Animals were derived from protocol (i), but none of embryos survived to day 40 from protocol (ii). Argumentation relating to in vivo matured oocytes is not followed (a distinguishing feature based on this is not in the claims anyway). Further, the data relating to calcium-activated oocytes cannot be ignored - this provides a working protocol with surviving embryos. Animals

clearly could be obtained derived from the calcium-activated cells.

D6 anticipates claims 10-12, 19-24, 29, 30, 36.

- **Inventive Step (Art.33(3) PCT)**

Only claims 5 and 32 appear to be novel. Claims 5 and 32 are novel due to the physical means used for oocyte activation. However, the oocyte activation protocols used by applicant and claimed were all known to the skilled person - physical methods just being a trivial selection from a number of known possibilities.

Hence, at present, no inventive subject-matter can be detected in the present application.

Applicants argumentation could not be followed.

- **Industrial Applicability (Art.33(4) PCT)**

For the assessment of the present claims 1-24 and 28-38 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Claims 1-24 and 28-38 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

VIII. **Certain observations**

Claims 25-27 include the manipulation of human embryos in their scope (since these claims have been amended in a manner which uncouples them from claims refering to non-human cells. This subject-matter is considered by the present IPEA to be contrary to morality and hence not allowable. Applicant is reminded to be very careful when dealing with such matters as inclusion of matter relating to human embryos can lead to major consequences should such matter proceed to grant in a subsequent regional procedure.

- **Clarity (Art.6 PCT)**

Claims 25-27 are unallowable product-by-process claims. The resulting embryo does not retain any features imparted by the particular method by which it was produced. Hence, these claims need to be deleted.

WHAT IS CLAIMED IS:

1. A method of preparing a reconstructed non-human oocyte by transferring cell or nucleus from germinal or somatic cells into an enucleated host oocyte, which comprises the steps of:
   a) activating said host oocyte;
   b) enucleating said activated host oocyte when said activated oocyte is undergoing the expulsion of a second polarbody or when said activated oocyte has expelled said second polarbody (Tel-II); and
   c) transferring nucleus from germinal or somatic cells into said enucleated host oocyte of step b) to obtain a reconstructed oocyte.

2. The method according to claim 1, wherein said transferred cell or nucleus is at nuclear stage G0, G1, S, G2, or M.

3. The method of claim 1, wherein said germinal or somatic cells of step c) are cultured prior to nucleus transfer.

4. The method of claim 1, wherein said oocyte of step a) is a secondary oocyte (M-II) and said activation is performed by artificial means selected from the group consisting of physical means and chemical means.

5. The method of claim 4, wherein said chemical means is ethanol or ionomycin.

6. The method of claim 4, wherein said physical means is selected from the group consisting of

electrical means, thermal means, and irradiation technology.

7. The method of claim 1, wherein step b) is performed after oocytes are cultured for a period of time sufficient to allow for at least partial extrusion of a second polarbody.

8. The method of claim 1, wherein step b) is performed with oocytes in a medium with cytosqueletal inhibitors.

9. The method of claim 7, wherein step b) is effected by microsurgically removing said second polarbody with a portion of the cytoplasm containing chromosomes surrounding said at least partially extruded second polarbody.

10. A method of reconstituting a non-human embryo, which comprises the steps of:

a) activating oocyte by artificial or natural means;

b) enucleating said activated oocyte when said activated oocyte is undergoing the expulsion of a second polarbody or when said activated oocyte has recently expelled second polarbody (Tel-II);

c) culturing germinal or somatic cell prior to nucleus transfer;

d) transferring a nucleus from said cell of step c) in said enucleated oocyte to obtain a reconstructed oocyte with a diploid chromosomal content; and

e) culturing *in vitro* said reconstructed oocyte and/or transferring said reconstructed

oocyte into a reproductive tract of a suitable surrogate mother to enable development into a non-human embryo.

11. The method according to claim 10, wherein said transferred cell or nucleus is at nuclear stage G0, G1, S, G2, or M.

12. The method of claim 10, wherein said oocyte of step a) is a secondary oocyte (M-II) and said artificial means is physical or chemical means.

13. The method of claim 12, wherein said chemical means is ethanol or ionomycin.

14. The method of claim 12, wherein said physical means is selected from the group consisting of electrical means, thermal means, and irradiation technology.

15. The method of claim 13, wherein step b) is performed after oocytes are cultured for a period of time sufficient to allow for at least partial extrusion of a second polarbody.

16. The method of claim 15, wherein step b) is performed with oocytes in a medium with cytosqueletal inhibitors.

17. The method of claim 15, wherein step b) is effected by microsurgically removing said second polarbody with a portion of the cytoplasm containing chromosomes surrounding said at least partially extruded second polarbody.

18. The method of claim 17, wherein step c) is effected by introducing a single cell containing a diploid nucleus into said enucleated oocyte by cell fusion or by microinjection.

19. The method of claim 10, wherein said non-human embryo develops into a non-human animal.

20. A method for production of a transgenic non-human embryo, which comprises the steps of:

a) activating oocyte by artificial or natural means;

b) enucleating said activated oocyte when said activated oocyte is undergoing the expulsion of a second polarbody or when said activated oocyte has recently expelled second polarbody (Tel-II);

c) culturing germinal or somatic cell prior to nucleus transfer;

d) transferring a transgenic nucleus from said cell of step c) transfected with a desired DNA construct in said enucleated oocyte to obtain a reconstructed oocyte with a diploid chromosomal content; and

e) culturing *in vitro* said reconstructed oocyte and/or transferring said reconstructed oocyte into a reproductive tract of a suitable surrogate mother to enable development into a non-human embryo.

21. The method according to claim 20, wherein said transferred cell or nucleus is at nuclear stage G0, G1, S, G2, or M.

22. The method according to claim 20, which further comprises developing said non-human embryo into a fetus.

23. The method according to claim 22, which further comprises developing said fetus into an offspring.

24. The method of claim 20, wherein said non-human embryo develops into a non-human animal.

25. A transgenic embryo obtained according to the method which comprises the steps of:

a) activating oocyte by artificial or natural means;

b) enucleating said activated oocyte when said activated oocyte is undergoing the expulsion of a second polarbody or when said activated oocyte has recently expelled second polarbody (Tel-II);

c) culturing germinal or somatic cell prior to nucleus transfer;

d) transferring a transgenic nucleus from said cell of step c) transfected with a desired DNA construct in said enucleated oocyte to obtain a reconstructed oocyte with a diploid chromosomal content; and

e) culturing *in vitro* said reconstructed oocyte and/or transferring said reconstructed oocyte into a reproductive tract of a suitable surrogate mother to enable development into a non-human embryo.

26. A transgenic fetus obtained according to the method which comprises the steps of:

a) activating oocyte by artificial or natural means;

b) enucleating said activated oocyte when said activated oocyte is undergoing the expulsion of a second polarbody or when said activated oocyte has recently expelled second polarbody (Tel-II);

c) culturing germinal or somatic cell prior to nucleus transfer;

d) transferring a transgenic nucleus from said cell of step c) transfected with a desired DNA construct in said enucleated oocyte to obtain a reconstructed oocyte with a diploid chromosomal content; and

e) culturing *in vitro* said reconstructed oocyte and/or transferring said reconstructed oocyte into a reproductive tract of a suitable surrogate mother to enable development into a non-human embryo.

27. A transgenic offspring obtained according to the method which comprises the steps of:

a) activating oocyte by artificial or natural means;

b) enucleating said activated oocyte when said activated oocyte is undergoing the expulsion of a second polarbody or when said activated oocyte has recently expelled second polarbody (Tel-II);

c) culturing germinal or somatic cell prior to nucleus transfer;

d) transferring a transgenic nucleus from said cell of step c) transfected with a desired DNA construct in said enucleated oocyte to

obtain a reconstructed oocyte with a diploid chromosomal content; and

e) culturing *in vitro* said reconstructed oocyte and/or transferring said reconstructed oocyte into a reproductive tract of a suitable surrogate mother to enable development into a non-human embryo..

28. A method of cloning a non-human animal by cell or nuclear transfer which comprises the steps of :

a) activating oocyte by artificial means;

b) enucleating said activated oocyte when said activated oocyte is undergoing the expulsion of a second polarbody or when said activated oocyte has recently expelled second polarbody (Tel-II);

c) culturing germinal or somatic cell prior to nucleus transfer;

d) transferring a diploid nucleus from said cell of step c) in said enucleated oocyte to obtain a reconstructed oocyte with a diploid chromosomal content; and

e) culturing *in vitro* said reconstructed oocyte and/or transferring said reconstructed oocyte into a reproductive tract of a suitable surrogate mother to enable development into a non-human embryo.

29. The method according to claim 28, wherein said transferred cell or nucleus is at nuclear stage G0, G1, S, G2, or M.

30. The method of claim 28, wherein said oocyte of step a) is a secondary oocyte (M-II) and said artificial means is physical or chemical means.

31. The method of claim 30, wherein said chemical means is ethanol or ionomycin.

32. The method of claim 30, wherein said physical means is selected from the group consisting of electrical means, thermal means, and irradiation technology.

33. The method of claim 28, wherein step b) is performed after oocytes are cultured for a period of time sufficient to allow for at least partial extrusion of a second polarbody.

34. The method of claim 30, wherein step b) is performed with oocytes in a medium with cytosqueletal inhibitors.

35. The method of claim 31, wherein step b) is effected by microsurgically removing said second polarbody with a portion of the cytoplasm containing chromosomes surrounding said at least partially extruded second polarbody.

36. The method of claim 32, wherein step c) is effected by introducing a single cell containing a diploid nucleus into said enucleated oocyte by cell fusion or by microinjection.

37. The method of claim 28, wherein said nucleus or cell of step c) is transgenic or non-transgenic.

38. The method of claim 28, wherein said non-human embryo develops into a non-human animal.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:
MG
SWABEY OGILVY RENAULT
1981, Avenue McGill College
Bureau 1600
Montréal, Québec H3A 2Y3
CANADA

SWABEY OGILVY RENAULT
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JUL 6 2001
A.M. 7 8 9 10 11 12 1 2 3 4 5 6 P.M.

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	28.06.2001
Applicant's or agent's file reference 10662-86PCT	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA00/00483	27/04/2000	28/04/1999

Applicant
UNIVERSITE DE MONTREAL et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	CLEERE, C Tel.+49 89 2399-8061

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
10662-86PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA00/00483	27/04/2000	28/04/1999
International Patent Classification (IPC) or national classification and IPC C12N15/00		
Applicant UNIVERSITE DE MONTREAL et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

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   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
15/11/2000	28.06.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Roscoe, R Telephone No. +49 89 2399 2554

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CA00/00483

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
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Drawings, sheets:

1/1	as originally filed				

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3. Additional observations, if necessary:
see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability: citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	5, 32
	No:	Claims	1-4, 6-31, 33-37
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-38
Industrial applicability (IA)	Yes:	Claims	25-27
	No:	Claims	1-24, 28-38

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

I. Basis

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Claims 1, 3, 5, 7-9, 13, 15-18, 28, 31, 33-35, 37 and 38

V. Reasoned statement on Novelty, Inventive Step and Industrial Applicability

- Novelty (Art.33(2) PCT)

D1 discloses enucleation technique where oocytes are activated using ethanol. This is followed by microsurgical removal of the telophase-stage chromatin in a small volume of cytoplasm adjacent to the second polar body. Following enucleation, a single blastomere derived from an in vitro produced morulla was injected into the perivitelline space of the enucleated oocyte. Fusion of the membranes was performed by electrical pulsing. The reconstructed oocytes obtained by the new technique produced developmentally competent reconstructed oocytes. Technique suggested to be useful for research and practice of mammalian cloning. D1 relates specifically to cloning of animals using early embryonic blastomeres. Such cells can be considered as having the status of both germinal or somatic. Unspecified periods of culture can obviously not establish a difference between this prior art and the present application since

unless specific times are defined which clearly differentiate from the prior art, the unspecified periods have to be considered as an unclear and thus irrelevant technical feature.

D1 anticipates claims 10-13, 15-18, 20, 21, 25, 28-31, 33-37.

D2 discloses enucleation technique which differs from the D1 technique essentially in that sequential calcium ionophore and cycloheximide treatment are used to activate oocytes. Further, it is specified explicitly that both recipient ooplast and donor blastomeres are probably effectively in S-phase. Suggests that use method to produce large numbers of identical progeny. D2 does not only relate to metaphase II enucleation. D2 compares enucleation efficiency before and after oocyte activation. Already in the abstract it is stated that 100% of chromatin material was found adjacent to the second polar body after the activation. This is clearly referring to oocytes that have proceeded beyond metaphase II to the telophase II at which the extrusion of the second polar body is evident. Applicants attention is also drawn to the first two paragraphs of the results section.

D2 anticipates claims 10-12, 15-18, 20, 21, 25, 28-30, 33-37.

D3 discloses a different enucleation technique. However anticipates claims 25-27, since embryos / animals / offspring are not distinguishable whether produced by method involving enucleation at 1st or 2nd polar body.

D4 discloses production of transgenic sheep. Anticipates claims 25-27.

D6 is only relevant to claims other than 1, 3, 5, 7-9, 13, 15-18, 28, 31, 33-35, 37 and 38 (see section on priority). D6 discloses electrofusion of transgenic somatic goat cells with oocytes which have been enucleated at Tel-II stage after activation by (i) calcium, (ii) ethanol. Animals were derived from protocol (i), but none of embryos survived to day 40 from protocol (ii). Argumentation relating to in vivo matured oocytes is not followed (a distinguishing feature based on this is not in the claims anyway). Further, the data relating to calcium-activated oocytes cannot be ignored - this provides a working protocol with surviving embryos. Animals

clearly could be obtained derived from the calcium-activated cells.

D6 anticipates claims 10-12, 19-24, 29, 30, 36.

- **Inventive Step (Art.33(3) PCT)**

Only claims 5 and 32 appear to be novel. Claims 5 and 32 are novel due to the physical means used for oocyte activation. However, the oocyte activation protocols used by applicant and claimed were all known to the skilled person - physical methods just being a trivial selection from a number of known possibilities.

Hence, at present, no inventive subject-matter can be detected in the present application.

Applicants argumentation could not be followed.

- **Industrial Applicability (Art.33(4) PCT)**

For the assessment of the present claims 1-24 and 28-38 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Claims 1-24 and 28-38 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

VIII. **Certain observations**

Claims 25-27 include the manipulation of human embryos in their scope (since these claims have been amended in a manner which uncouples them from claims refering to non-human cells. This subject-matter is considered by the present IPEA to be contrary to morality and hence not allowable. Applicant is reminded to be very careful when dealing with such matters as inclusion of matter relating to human embryos can lead to major consequences should such matter proceed to grant in a subsequent regional procedure.

- **Clarity (Art.6 PCT)**

Claims 25-27 are unallowable product-by-process claims. The resulting embryo does not retain any features imparted by the particular method by which it was produced. Hence, these claims need to be deleted.